Functional differences between site-specific osteoclasts

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Lysosomal-associated membrane protein-2 (LAMP-2) is involved in osteoblast-induced osteoclastogenesis

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ABSTRACT

Multinucleated osteoclasts are specialized cells with the unique capacity to resorb bone. To perform this activity, the osteoclast has a specialized membrane area, the ruffled border. This membrane bears similarities with lysosomal membranes. Two lysosome associated membrane proteins (LAMP-1 and -2) represent about half of the total amount of proteins associated with the lysosomal membrane and LAMP-2 was shown to be highly expressed in the ruffled border membrane of the osteoclast. To elucidate its possible function in the formation and activity of osteoclasts, we analyzed bones and osteoclasts of LAMP-2-deficient mice, and the formation of osteoclasts *in vitro.* We found that the bone volume (BV/TV) of these mice was lower compared to wild type mice of comparable age. *In situ* osteoclasts of LAMP-2 deficient mice displayed a normal ruffled border but contained fewer vesicles. M-CSF and RANKL driven osteoclastogenesis cultures showed a higher number of osteoclasts in LAMP-2 deficient cultures compared to wild type cultures. Surprisingly, a near complete absence of osteoclast formation was found when precursors were co-cultured with LAMP-2 deficient osteoblasts. FACS analysis revealed that membrane-bound RANKL was strongly decreased on LAMP-2 deficient osteoblasts. These results show that osteoblastic LAMP-2 proved to be essential for osteoblast-induced osteoclast formation. The findings suggest that LAMP-2 plays a role in the expression of RANKL on the membrane of osteoblasts.

INTRODUCTION

The multinucleated osteoclast is the only cell type equipped to resorb bone. To perform this activity, the osteoclast has a unique membrane area where protons are released to lower the pH as well as proteolytic enzymes to digest the matrix. This area is called the ruffled border. The membrane of this ruffled border has a unique composition. It is formed by fusion of lysosomal vesicles resulting in a membrane with properties also found in lysosomal membranes [1].

The composition of the lysosomal membrane is different from all other membranes in eukaryotic cells due to an extremely high carbohydrate content, a characteristic phospholipid composition, the presence of cholesterol, the presence of various ion pumps, and the presence of unique membrane proteins including the lysosome associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2). These latter proteins represent about 50% of the total amount of lysosomal membrane proteins [2, 3].

Lysosomes are defined as acidic hydrolase-rich cell organelles [4, 5], and are involved in destruction or recycling of cellular and extracellular components. These components are delivered to the lysosome via phagocytic, endocytic or autophagic vacuoles. The single membrane of the lysosome serves as a barrier to keep the acidic environment separate from the rest of the cell's cytoplasm. The LAMPs contribute to the fusion between lysosomes and endosomes or other organelles [6]. In addition, the LAMPs have been associated with inactivation of pathogens, down regulation of surface receptors, repair of plasma membranes and loading of processed antigens onto MHC class II molecules [7].

After the generation of LAMP-1 and LAMP-2 knock-out mice it became clear that LAMP-1 and- 2 share some functions [8]. An upregulation of LAMP-2 was found in LAMP-1 deficient mice, which might explain why these mice were viable and fertile and none of the lysosomal enzyme activities appeared to be affected in these mice [9]. In contrast to the relatively mild phenotype of the LAMP-1 deficient mice, LAMP-2 deficient mice showed a more severe phenotype. Huge accumulations of autophagic vacuoles were found in cells of the liver, pancreas, spleen, skeletal muscle, heart, lymph nodes, and in neutrophils. A high number of LAMP-2 deficient mice died prematurely, within 40 days after birth, probably due to an extensive accumulation of autophagic vacuoles [10, 11]. Recently, it became clear that LAMPs are involved in processes such as chaperone mediated autophagy [12, 13].

Beertsen et al. [14] showed that mice deficient for LAMP-2 develop severe periodontitis due to an impaired phagosomal maturation of neutrophils. The fusion between lysosomes and phagosomes was hampered resulting in a defective clearance of the pathogens.

In spite of the presence of LAMP-2 in the ruffled border of osteoclasts [15-17], a possible role of this protein with respect to osteoclast function has not been elucidated. In an attempt to shed light on this, we analyzed bones, osteoclasts and osteoblasts of LAMP-2 deficient mice. Furthermore, we analyzed the role of LAMP-2 in osteoclast formation in two different ways: (1) by a cytokine approach using M-CSF and RANKL, and (2) by co-culturing bone marrow cells with osteoblast-like cells. Since it is known that heterogeneity exists between osteoclasts present at the different bone sites [18-21] bone marrow cells and osteoblasts were isolated from long bone and calvaria to explore whether osteoclast precursors from the different bone sites were differently affected in their osteoclast potential due to LAMP-2 deficiency.

MATERIALS & METHODS

Mice

LAMP-2 deficient mice (C57B6x129SV) were generated as described previously [10]. Experiments were approved by the IACUC (The Institutional Animal Care and Use Committee) of the VU University (Amsterdam, The Netherlands). The mice used in the different experiments were males of 7-14 weeks old. Since the LAMP-2 gene is localized on the X-chromosome, the male knock-out mice are indicated as LAMP-2-/y. Per experiment all genotypes were age-matched. The mice were sacrificed and tibiae and calvariae were isolated and used for bone marrow or osteoblast isolation or the bones were fixed for electron microscopy or µCT (see below).

Micro CT

To study the effect of LAMP-2 deficiency on bone parameters, such as trabecular volume and bone volume, intact tibiae and calvariae were examined from age-matched wild-type and LAMP-2 deficient mice. The bones were fixed in fixative (see microscopy) and cleaned of soft tissue associated with the bones. The bones

were scanned in a micro-computed tomography apparatus (μCT20, Scanco Medical AG, Zurich, Switzerland) as described in Giesen et al.[22]

Microscopy

Tibiae and calvariae were isolated and fixed for 48 h at room temperature in 4% formaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After removal of soft connective tissue from the bone samples and μCT analysis the bones were decalcified for two weeks in 0.1 M EDTA and 1% glutaraldehyde in cacodylate buffer, washed in buffer, postfixed in 1% OsO4, washed in buffer again, dehydrated through a graded series of ethanol, and embedded in epoxy resin (LX-112). Semi-thin sections of 1 μm thickness were cut with a diamond knife, stained with methylene blue and used for (a) general morphology, (b) assessment of bone density, and (c) analysis of osteoclasts (number, and the presence of ruffled border) and osteoblasts. Ultrathin sections were made with a diamond knife, stained with uranyl and lead and examined in a Philips CM10 electron microscope. For the analysis of vesicles present in the osteoclasts low power electron micrographs were made (6800x). A point-counting method was performed using a double latticed grid according to Weibel [23].10-15 osteoclasts of each bone type and genotype were micrographed and used to assess the volume density of intracellular vesicles of the osteoclasts. Data were expressed as percentage of total cytoplasm.

Osteoclast generation using M-CSF and RANKL

Osteoclasts were generated as described [24]. Briefly, for each experiment, age-matched wild type and LAMP-2-/y mice were sacrificed with a peritoneal injection of sodium pentobarbital (0.1 ml Euthestate, Ceva Sante Animale, Naaldwijk, The Netherlands). Tibiae and calvariae were dissected and cleaned of soft tissue, and ground separately in a mortar with culture medium that consisted of α-MEM (Invitrogen, Paisley, UK) supplemented with 5% fetal calf serum (HyClone, Logan, UT, USA), 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (Antibiotic Antimycotic solution, Sigma, St. Louis, MO, USA), and heparin (170 IE/ml; Leo Pharmaceutical Products B.V., Weesp, The Netherlands). The cell suspensions were aspirated through a 21-gauge needle and filtered over a 70 μm pore-size Cell Strainer filter (Falcon/ Becton Dickinson, Franklin Lakes, NJ, USA). Cells were washed twice in culture medium, centrifuged (5 min, 200 g), and plated

in 96-well flat-bottom tissue-culture-treated plates (Cellstar, Greiner Bio-One, Monroe, NC, USA) at a density of 1.10^5 cells per well; alternatively, cells were seeded on bovine cortical bone slices of 650 µm in thickness.

Cells were cultured in 150 µl culture medium containing 30 ng/ml recombinant murine M-CSF (R&D systems, Minneapolis, MI, USA) and 20 ng/ml recombinant murine RANKL (R&D systems). Culture media were refreshed every fourth day. After six days of culture, wells were washed with PBS and either fixed in 4% PBS buffered formaldehyde and stored at 4 ºC, used for tartrate-resistant acid phosphatase (TRACP) staining, or dissolved in RNA lysis buffer (see below) and stored at -80 ºC until RNA isolation. The wells with the bone slices were stored in milliQ water at 4ºC for the analysis of bone resorption.

Isolation of osteoblast-like cells

Tibiae of wild type and LAMP-2 deficient mice were cleaned of soft tissue and the bones were cut into small pieces. The fragments were incubated for 2 hours at 37º C with 2 mg/ml collagenase II (Sigma)[25]. These fragments were subsequently rinsed and placed in 25 cm^2 culture flasks (Cellstar, Greiner Bio-One) with 5 ml DMEM (Invitrogen) supplemented with 10% FCS (HyClone) and 1% antibiotic-antimycotic solution in a humidified atmosphere of 5% CO_2 in air at 37°C. After a couple of days cells grew out of the bone fragments. When the cells were confluent (after 3-4 weeks) they were collected by the use of 0.25% trypsin and 0.1% EDTA (pH 7.3), and transferred to 175 cm^2 culture flasks (Cellstar, Greiner Bio-One), designated as 'passage one'. The cells of this passage and subsequent passages showed a typical trapezoidal shape. For the subsequent experiments we used cells of passage 2-4.

Co-culture

Osteoblast-like cells obtained from wild type and LAMP-2 deficient mice were plated in a 96 well plate at a density of 8 x 10^3 cells/well in α-MEM (Invitrogen) supplemented with 10% FCS and 1% antibiotic-antimycotic solution and 10 nM vitamin D (Sigma) in a humidified atmosphere of 5% CO_2 in air at 37°C. After their attachment during a 24 hour pre-culture, bone marrow cells from wild type mice and LAMP-2 deficient mice were added in a concentration of 1 x10^5 cells per well. Cells were cultured together for 9 days in α-MEM (Invitrogen) supplemented with 10% FCS, 1% antibiotic antimycotic solution and 10 nM vitamin D.

TRACP activity in cells

TRACP staining was carried out using the Acid Phosphatase Leukocyte (TRACP) Kit from Sigma according to the manufacturer's instructions. The number of multinucleated cells on bone and on plastic were counted. The number of cells with 3 or more nuclei was assessed and the cells were grouped into one of the following categories: (i) cells with 3-5 nuclei, (ii) cells with 6-10 nuclei, and (iii) cells with more than 10 nuclei.

Quantitative RT-PCR.

RNA from cultured bone marrow cells, osteoblasts and co-cultures was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After measuring RNA concentration with a multilabel plate reader (Synergy HT, BioTek Instruments, Bad Friedrichshall, Germany), 100 ng RNA was reverse transcribed to cDNA for real-time quantitative PCR (qPCR) with the superscript Vilo cDNA synthesis kit (Invitrogen) or first strand synthesis kit (Fermentas, Thermo Fisher Scientific Inc., Waltham, MA USA). The reactions were performed with the ABI PRISM 7000 (Applied Biosystems) by using 5 ng cDNA and 300 nM of each primer in a total volume of 15 µl containing SYBR Green PCR Master Mix (SYBR Green I Dye, AmpliTaq Gold DNA polymerase, and dNTPs with dUTP instead of dTTP, Applied Biosystems), according to the manufacturer's instructions. Samples were normalized for the expression of hypoxanthine guanine phosphoribosyl transferase (HPRT) by calculating the ΔCt (Ct gene of interest – Ct HPRT); expression of the different genes is given as 2^ –(ΔCt). The primers used for the detection of the various genes are indicated in Table I.

Table I: primers used for Quantitative PCR

Primer	Sequence 5'-3'
HPRT	Fw: CCTAAgATgAgCgCAAgTTgAA Rv:CCACAggACTAgAACACCTgCTAA
RANKL	Fw: CTgAggCCCAgCCATTTg RV: ggAACCCgATgggATgCT
DC-STAMP	Fw: TgTATCggCTCATCTCCTCCAT Rv: gACTCCTTgggTTCCTTgCTT
v-ATPase(d2)	Fw: TggAACTAgCTCCTAACCACCT Rv: AgTTgTAAgCAgACCCTgTtgg
OPG	Fw: TCCggCgTggTgCAA Rv: ATACAgggTgCTTTCgATgAAgTC
CXCL12	FW: TgTgCATTgACCCgAAATTA RV: TCTCACATCTTgAgCCTCTTgT

Immunolocalization of LAMP-2 and RANKL

The osteoclast cultures (M-CSF + RANKL cultures) were fixed after 6 days of culture and the osteoblast cultures after 10 days. For LAMP-2 localization (osteoclasts) the cells were fixed for 10 min. with 4% PBS buffered formaldehyde and subsequently washed with PBS. For RANKL localization in osteoblasts 0.1 % triton-X100 was added to the fixative to permeabilize the cells. Before incubation with the primary antibodies non-specific binding was blocked with "image it Fx signal enhancer" (Invitrogen/Molecular Probes, Carlsbad, CA) for 30 min at ambient temperature. Primary antibodies used were rat monoclonal antibody against mouse LAMP-2 (ABL93 1:200 dilution, this antibody was developed by J.T. August, and obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH, and maintained at The University of Iowa, Department of Biology, Iowa City, IA) and RANKL (goat anti-mouse 1:20, R&D). Isotype IgG or non-immune polyclonal goat serum (DAKO, Glostrup, Denmark) were used as negative controls. Incubations with the first antibodies were overnight at 4°C.

Bone slices with osteoclasts or wells with osteoblasts were washed three times with PBS and incubated for 60 min. with the secondary antibody chicken-anti-rat-Alexa 488 (Invitrogen) for LAMP-2 localization and rabbit-anti-goat-Alexa 488

conjugated antibody (Invitrogen) for RANKL localization. Following three PBS washes the LAMP-2 labeled osteoclast cultures were stained for F-actin using Alexa 633-phalloidin (Invitrogen) as described previously [26]. Finally, after washing, a drop of Vectashield (Vector, Burlington, Ontario, Canada) was added to prevent quenching; the vectashield was supplemented with propidium iodide to stain nuclei. Image stacks were generated with a confocal laser scanning microscope (Leica microsystems, Wetzlar, Germany) using an argon laser (for Alexa 488 and propidium iodide) and a helium laser (for Alexa 633) and the expression of RANKL and LAMP-2 in osteoblasts was visualized with a Leica IMDR converted fluorescence microscope equipped with a digital camera (Leica DFC 320).

FACS analysis of RANKL

Cultured osteoblasts, from WT and LAMP-2-/y mice, of passage 4 were removed from 75cm^2 culture flasks with cell dissociation solution (Sigma C5914). Cells were labeled with RANKL antibody (goat anti mouse RANKL R&D in a dilution of 1:20/10^5 osteoblasts) for 2 hours at 4ºC and subsequently incubated with a secondary antibody (rabbit anti goat alexa-488 (Invitrogen, 1:200)) for 30 min. at 4ºC. The samples were analyzed in a flow cytometer (C6 Flow Cytometer, Accuri, Cambs, UK). The data were calculated by subtraction of the non-immune serum fluorescence (goat IgG, Dako).

Statistics

A one-sample t-test was used to analyze isolated bone marrow cells, gene expression, bone resorption, TRAcP secretion and osteoclast formation in co-cultures. Differences were considered significant at $p<0.05$. Data are expressed as mean values of at least 3 measurements ± SEM.

RESULTS

Bone volume and shaft thickness is lower in LAMP-2-/y bones

Micro CT analysis revealed a comparable lower bone thickness in long bones and calvaria bone of the LAMP-2-/y mice (Figure 1A). The bone volume per total volume (BV/TV) was lower in calvaria and long-bones of the LAMP-2 -/y mice (Figure 1B).

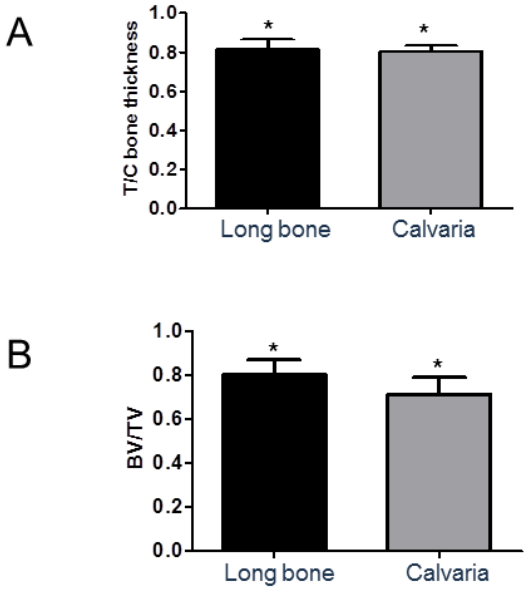


Figure 1. Thinner bone in LAMP-2 -/y bones. Bone thickness (A) and bone volume per total volume (B; BV/TV; n=4 for long bone and n=6 for calvaria) of LAMP-2 -/y bones expressed as LAMP-2 -/y over wild type (T/C). (n=5 for long bone and n=4 for calvaria) *p<0.05.

LAMP-2-/y osteoclasts are active

Osteoclasts were present in the LAMP-2-/y mice (Figure 2A). Standardized made micrographs of the bones were used to count the number of osteoclasts. Their number present in the shaft was comparable with wild type mice (Figure 2B). Osteoclasts of the deficient mice revealed, comparable to the wild type mice, the presence of a clear zone (Figure 3; marked with an asterisk) and a ruffled border.

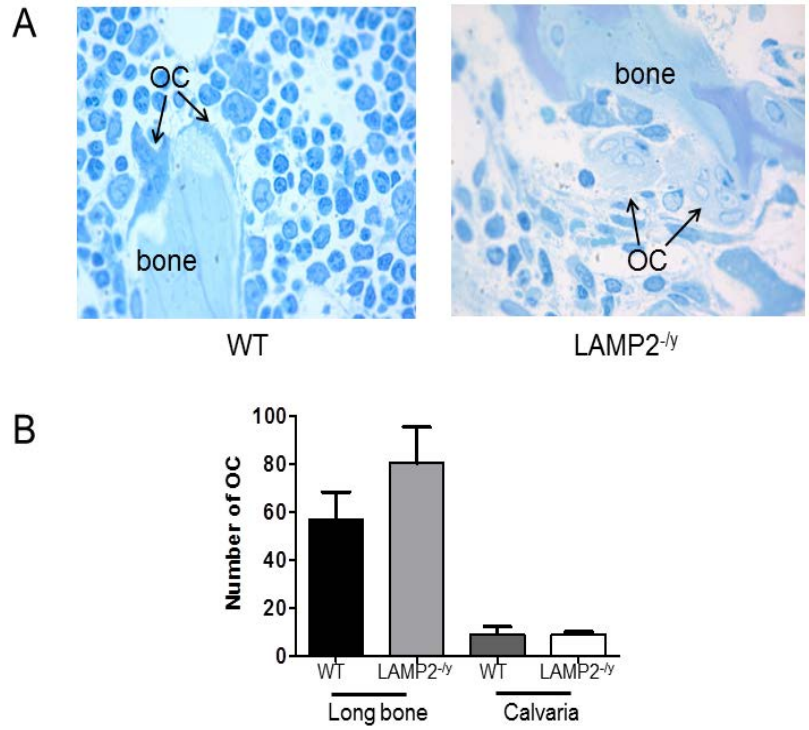


Figure 2. Comparable numbers of osteoclasts in LAMP-2 -/y mice. A. Micrographs of osteoclasts (OC) present in the bones of WT and LAMP-2 -/y mice. B. Comparable numbers of osteoclasts were found in WT and LAMP-2 -/y bones. Bones of 3 mice per genotype were counted.

Immunolocalization of LAMP-2 in osteoclasts and osteoblasts

Localization of LAMP-2 in *in vitro* generated osteoclasts revealed that the protein was localized primarily in the ruffled border. In the micrograph (Figure 4A) most of the green fluorescence, indicating the presence of LAMP-2, was found to be encircled by blue label which delineates the actin ring. LAMP-2-/y osteoclasts were negative for LAMP-2-/y label (not shown).

In wild type osteoblasts LAMP-2 is highly expressed throughout the cell (Figure 4B). The LAMP-2-/y osteoblasts did not show any positive staining (Figure 4B).

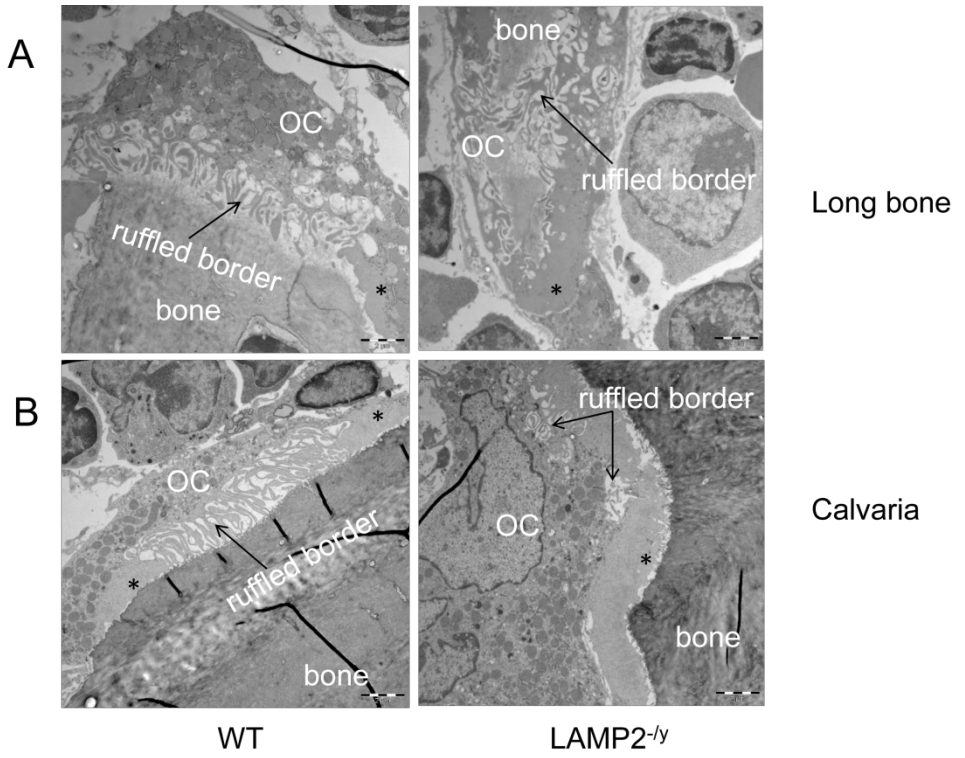


Figure 3. LAMP-2 -/y osteoclasts have a ruffled border. Electron micrographs of osteoclasts present in long bone (A) and calvaria (B). Wild type and LAMP-2 -/y osteoclasts (OC) express a ruffled border (arrow). Also a clear zone is present (asterisk).

Osteoclasts are readily formed from LAMP-2-/y bone marrow with M-CSF and RANKL

Next, we analyzed the formation of osteoclasts. Therefore, we cultured bone marrow cells from tibiae and calvariae in the presence of M-CSF and RANKL. Calvaria from LAMP-2-/y mice contained 4 times more marrow cells than wild type calvaria (not shown). From long bones similar numbers of bone marrow cells were isolated from wild-type and LAMP-2 -/y.

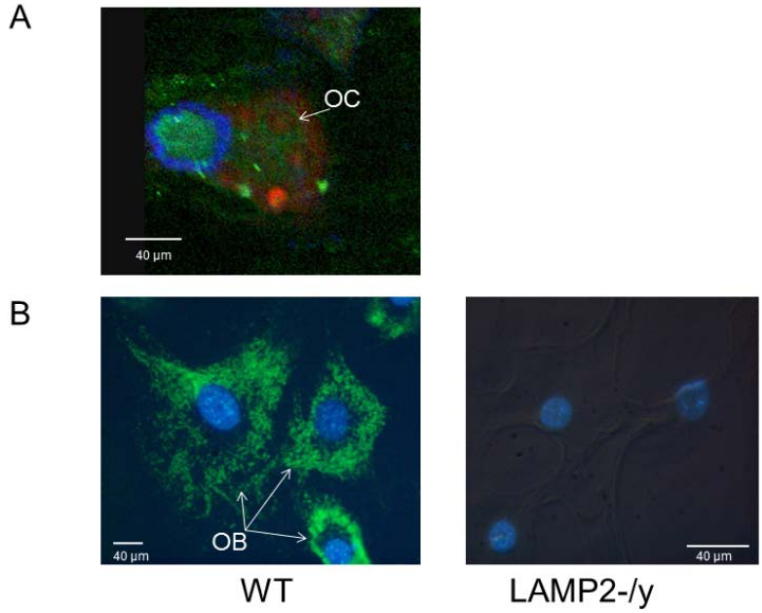


Figure 4. LAMP-2 localization in osteoclasts and osteoblasts. **(**A) LAMP-2 localization in osteoclasts (OC). Wild type osteoclasts were generated with M-CSF and RANKL on bone slices. Nuclei were stained with propidium iodide (red), LAMP-2 was visualized with alexa-488 (green), and actin was visualized with phalloidin-alexa 633 (blue). Note that the green LAMP-2 label is predominantly present in areas surrounded by an actin ring. (B) LAMP-2 localization in wild type and LAMP-2-/y osteoblasts (OB). The green LAMP-2 label is present throughout the wild type cell and completely absent in the LAMP-2-/y cells. Nuclei were stained with DAPI (blue).

After 6 days of culture the cells were stained for TRAcP activity and the number of multinucleated cells was assessed (Figure 5A,B). In all cultures multinucleated osteoclasts were formed. In the LAMP-2-/y calvaria cultures the number of cells was equal or even somewhat higher (Figure 5A,B).

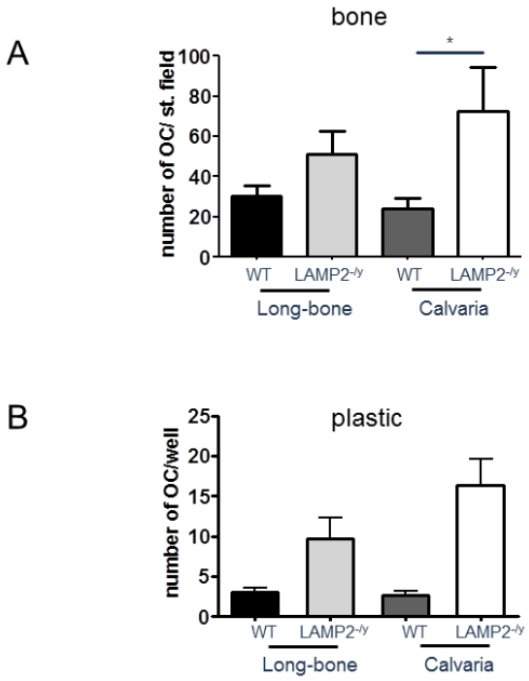


Figure 5. Osteoclast formation in M-CSF and RANKL stimulated bone marrow cultures. Number of multinucleated cells, with more than 2 nuclei, formed after 6 days of culture with M-CSF and RANKL from calvaria and long bones of wild type and LAMP-2 -/y mice. In (A) the number of multinucleated cells generated on bone is shown, and(B) shows the number of multinucleated cells generated on plastic (n=6 *p<0.05).

Osteoblastic LAMP-2 is essential for osteoclastogenesis

It is known that osteoclasts differentiate by a direct interaction between osteoblasts and osteoclast precursors [27, 28]. We therefore isolated osteoblasts from wild type and LAMP-2 deficient mice and co-cultured these cells with osteoclast precursors obtained from the two phenotypes.

In co-cultures with wild type osteoblasts multinucleated osteoclasts were readily formed. This was irrespective of the genetic background of the bone marrow cells and irrespective of the skeletal site the osteoblasts were isolated from. However, with osteoblasts obtained from LAMP-2-/y mice a striking effect was noted: hardly

any osteoclasts were formed (Figure 6). This was apparent for osteoblasts obtained from both bone sites and irrespective of the source of the precursors (wild type or LAMP-2-/y). These findings indicate that expression of LAMP-2 by osteoblasts is essential for osteoblast-driven osteoclast differentiation.

Also, the number of osteoclasts formed by the cells obtained from the two different bone sites proved to be different. In the co-cultures with calvarial cells four times more osteoclasts were formed compared to the cultures with long bone cells (compare Figure 6A and B; $p<0.05$).

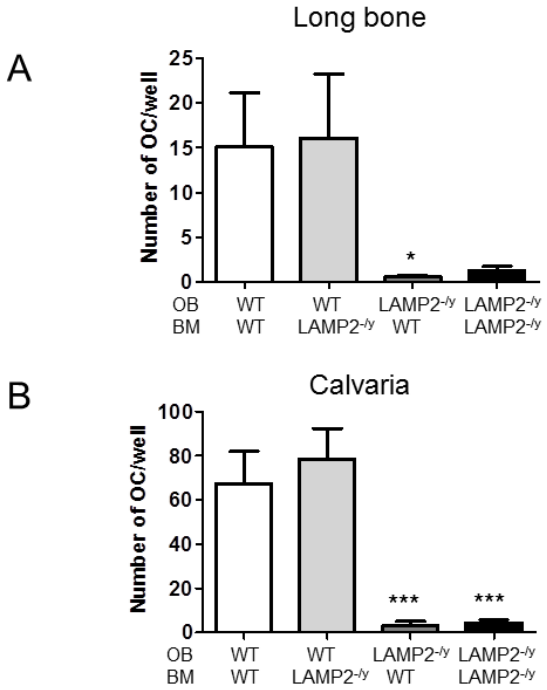


Figure 6. Osteoblasts of LAMP-2 deficient mice do not induce osteoclast formation.
Osteoblasts (OB) and bone marrow cells (BM) from long bone (A) and calvaria (B) of wild type (WT) and LAMP-2 -/y mice were co-cultured for 10 days. Wild type osteoblasts induced the formation of multinucleated osteoclasts. This occurred with bone marrow cells of both genotypes (WT or LAMP-2-/y). Hardly any osteoclasts were generated in the co-cultures with LAMP-2-/y osteoblasts (n= 9, *p< 0.05, ***p< 0.001).

Expression of osteoclast related genes is affected in the co-cultures

We then evaluated osteoclast and osteoblast gene expression. The data shown are only from co-cultures of wild type bone marrow cells with osteoblasts of LAMP-2-/y or of wild type mice since comparable results were found when the bone marrow cells were from LAMP-2-/y genotype. The expression of v-ATPase (d2 subunit) and DC-STAMP was lower in co-cultures with LAMP-2-/y osteoblasts compared to the wild-type co-cultures (Figure 7A, B). The lower expression of v-ATPase was only significant for calvaria cells and not for those obtained from long bone.

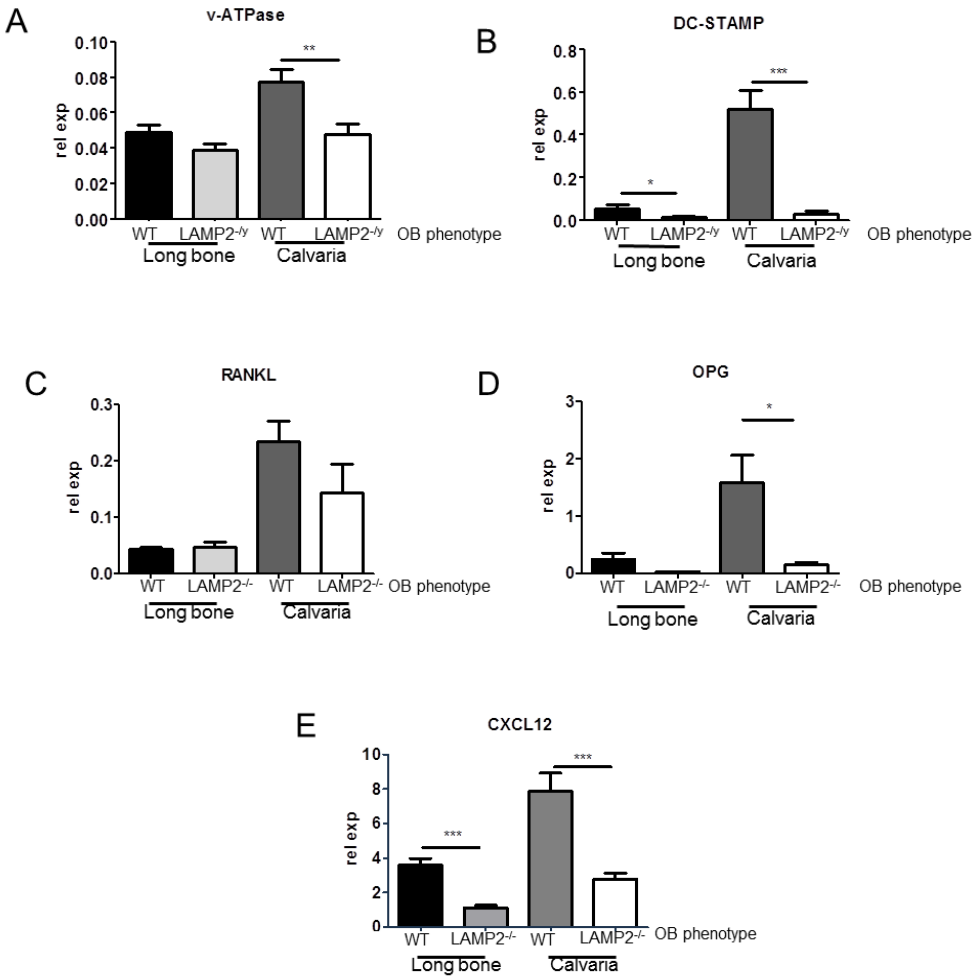


Figure 7. Expression of DC-STAMP, v-ATPase, and CXCL12 is lower in co-cultures with LAMP-2 -/y osteoblasts. QPCR analysis was performed after 10 days of co-culture. Since no differences were found between the expression of bone marrow cells obtained from wild type or LAMP-2-/y mice, the data for co-cultures with wild type and LAMP-2-/y bone marrow and LAMP-2-/y were combined. Cultures with osteoblasts of LAMP-2-/y origin revealed a lower expression of v–ATPase (A) in LAMP-2 -/y calvaria cultures and DC-STAMP (B) for both types of cultures (n=18 *p< 0.05, **p< 0.01, ***p<0.001). C. RANKL expression was not significantly changed in the co-cultures with LAMP-2-/y osteoblasts (n=18). D. OPG expression is lower in the co-cultures with LAMP-2 -/y osteoblasts, however only significant for calvaria (n=5, *p<0.05). The CXCL12 (E) expression is significantly lower in the co-cultures with LAMP-2-/y osteoblasts (n=18, ***p<0.0005)

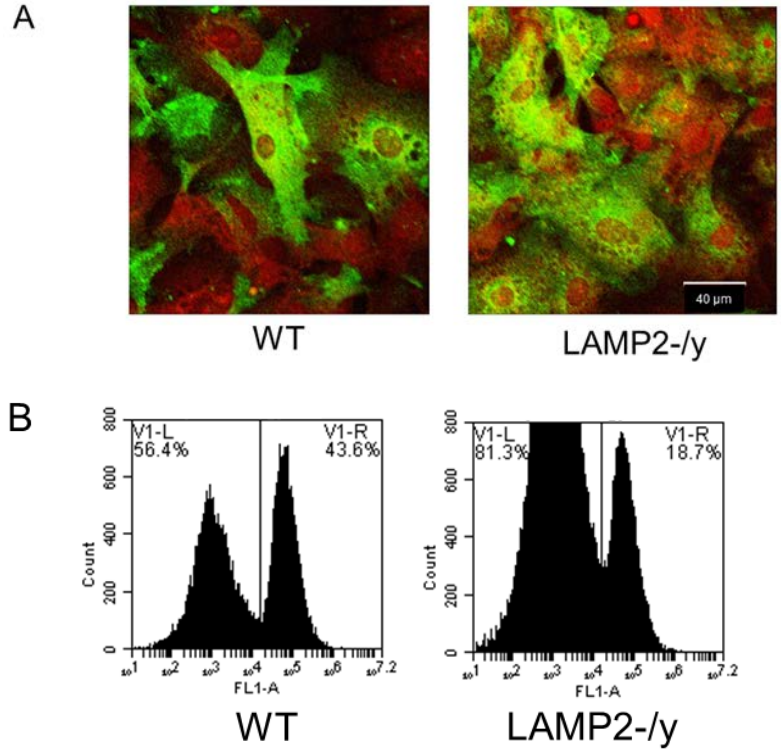
Expression of osteoblast related genes is affected in the co-cultures

No significant differences were found in RANKL expression by comparing co-cultures from long bone or those from calvaria (Figure 7C). However, a comparison between calvaria and long bone revealed a much higher expression of RANKL expression by the calvaria cultures. The expression of OPG was higher in co-cultures with the LAMP-2-/y osteoblasts but this was statistically significant only for the calvaria cells (Figure 7D). The expression of CXCL12 (or SDF-1) was significantly lower in the co-cultures with LAMP-2-/y osteoblasts (Figure 7E).

Lower RANKL surface expression of LAMP-2-/y osteoblasts

Since osteoclasts were formed in high numbers when RANKL was added to bone marrow cultures, and we did not find osteoclast formation in the co-cultures, we considered a hampered RANKL expression by osteoblasts. It is known that osteoblast-osteoclast communication depends largely on RANK-RANKL binding. Immunolocalization of RANKL showed a strong labeling of this protein in wild type as well as LAMP-2-/y osteoblasts. Not all cells, however, were labeled. About 50% of the cells were strongly labeled whereas the others were negative. This was apparent for osteoblasts obtained from both phenotypes (Figure 8A). Since we used Triton in these localization experiments it was not possible to distinguish between plasma membrane associated label or label localized intracellular.

To investigate the membrane expression of RANKL we performed a FACS analysis. This revealed that the percentage of cells expressing membrane RANKL was much lower for the LAMP-2-/y osteoblasts (Figure 8B). In wild-type cultures 52% ± 7 of the cells were positively labeled; in the LAMP-2 -/y cultures this was only 19% ± 1. Moreover, the amount of RANKL present on the positively labeled cells was 6 times lower for the LAMP-2-/y cells.



OB	% unlabeled cells	% labeled cells	Mean fluoresence
WT	49.6 ± 6	51.6 ± 7	20.220
LAMP-2 -/y	80.8 ± 0.8	19.2 ± 0.8	3.193

Figure 8. Immunolocalization of RANKL in wild-type and LAMP-2 -/y osteoblasts.
(A) WT and LAMP-2 -/y were labeled for RANKL and visualized with alexa-488 (green), the nuclei were stained with propidium iodide (red). About 50% of the osteoblasts from both, WT and LAMP-2 -/y mice are intensely labeled for RANKL.(B) FACS analysis showed that not all cells are positively labeled for RANKL. 52% of WT osteoblasts were positive for RANKL, whereas only 19% of the LAMP-2 -/y osteoblasts expressed RANKL on their membrane. WT osteoblasts contained about a 6-fold higher level of membrane-bound RANKL compared to the LAMP-2 -/y cells.

DISCUSSION

An intriguing finding of the present study was the involvement of LAMP-2 in osteoblast-mediated osteoclast formation. The osteoblasts from the deficient mice lacked the capacity to induce osteoclast formation. This effect was clearly related to the osteoblasts and not to the osteoclast precursors. LAMP-2 deficient osteoclast precursors not only formed osteoclasts with wild type osteoblasts but also in the presence of M-CSF and RANKL osteoclasts were readily generated. In an attempt to come up with an explanation for this finding we analyzed the expression of RANKL by the osteoblasts. We found that deficient osteoblasts expressed 6-fold less RANKL on their plasma membrane, thus making it plausible that this caused the almost completely inhibited osteoclastogenesis. How can such a low expression on the membrane be explained? Others showed that most of newly synthesized RANKL is not immediately transported to the membrane, but stored in LAMP-1 containing vesicles [29-31]. It is not unlikely to assume that these vesicles also expressed LAMP-2, thus making it plausible that in the absence of this protein RANKL transportation is hampered. In line with such a role, data presented by Zhou et al. [32] indicate that LAMP-2 plays a role in presentation of antigens via MHC II.

A hampered RANK-RANKL interaction in these co-cultures can also explain the lower expression of the osteoclast related genes v-ATPase and DC-STAMP. Yagi et al. [33] showed that the transcription pathway of these genes is upregulated upon RANK-RANKL binding.

Interestingly, at least in co-cultures with LAMP-2-/y calvaria osteoblasts, osteoprotegerin (OPG) a decoy receptor for RANKL is down regulated. Lower membrane RANKL expression by these osteoblasts can be seen in the context of findings by Aoki et al.[29]. They showed that two pathways exist for RANKL transport to the membrane: a direct minor pathway and an indirect major pathway via secretory lysosomes. This latest pathway is upregulated by binding of RANKL (expressed via the minor pathway) to RANK on pre-osteoclasts. The major pathway was suggested to regulate osteoclastogenesis. They suggested that RANKL and OPG form a complex in the Golgi apparatus, which is needed for proper transport and trimerisation of RANKL at the membrane. It is possible that the minor pathway functions normally in the osteoblasts, but that the major pathway is blocked in the absence of LAMP-2. We suggest that the active trimer

form of RANKL is not transported to the membrane and subsequently osteoclastogenesis is severely hampered [29, 30].

It was of interest to note that the expression of CXCL12 mRNA was lower in the co-cultures with deficient osteoblasts. CXCL12 is a chemokine expressed and secreted by osteoblasts and in combination with its receptor, CXCR4, present on bone marrow cells, it is involved in migration and survival of osteoclast precursors [34]. In addition to the lower expression of RANKL, a lower membrane expression of CXCL12 might also result in the lower osteoclastogenesis potential of the co-cultures.

In spite of the fact that osteoclasts were hardly formed in the co-cultures with deficient osteoblasts, the number of osteoclasts *in vivo* appeared unaffected. We assume involvement of other pathways or other cell types in the *in vivo* formation of osteoclasts, such as bone lining cells and/or osteocytes. Recently it has been shown that RANKL produced by osteocytes is a major trigger for osteoclastogenesis [35, 36].

Remarkable is that a lysosome-rich cell as the osteoclast appears unaffected in the LAMP-2 deficient mice, whereas the osteoblast is [37]. Recently it was shown that in the lysosomal storage disease, mucolipidosis II, which is caused by an incorrect localization of some lysosomal proteins, due to the absence of their lysosomal transport protein mannose-6-phosphate receptor, the osteoclasts were also not affected, but their formation was enhanced due to altered signals produced by the osteoblasts [37, 38]. Thus, also under these conditions osteoblasts, but not osteoclasts, were affected; a finding in line with our findings. Why a lysosome-rich cell, like the osteoclast does not express a clear phenotype is not known and needs further investigation. It is possible that lysosomes present in different cell types such as osteoblasts, osteoclasts and neutrophils express different functions. Lysosomes have been shown to differ in their functional properties like secretion of proteases, plasma membrane repair, signaling and energy metabolism [39].

In conclusion, our results demonstrate a crucial role played by LAMP-2 in osteoblast-mediated osteoclast formation. We propose that LAMP-2 is an important protein needed for transportation of RANKL to the osteoblast membrane, thereby facilitating osteoclastogenesis.

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REFERENCES

1. **Baron R, Neff L, Louvard D, Courtoy PJ.** Cell-mediated extracellular acidification and bone resorption: evidence for a low pH in resorbing lacunae and localization of a 100-kD lysosomal membrane protein at the osteoclast ruffled border. J.Cell Biol. 101[6], 2210-2222. 1985.
2. **Eskelinen EL.** Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy. Mol.Aspects Med. 27[5-6], 495-502. 2006.
3. **Hunziker W, Simmen T, Honing S.** Trafficking of lysosomal membrane proteins in polarized kidney cells. Nephrologie 17[7], 347-350. 1996.
4. **Luzio JP, Pryor PR, Bright NA.** Lysosomes: fusion and function. Nat.Rev.Mol.Cell Biol. 8[8], 622-632. 2007.
5. **Appelqvist H, Waster P, Kagedal K, Ollinger K.** The lysosome: from waste bag to potential therapeutic target. J.Mol.Cell Biol. 5[4], 214-226. 2013.
6. **Saftig P, Schroder B, Blanz J.** Lysosomal membrane proteins: life between acid and neutral conditions. Biochem.Soc.Trans. 38[6], 1420-1423. 2010.
7. **Jimi E, Nakamura I, Duong LT, Ikebe T, Takahashi N, Rodan GA, Suda T.** Interleukin 1 induces multinucleation and bone-resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells. Exp.Cell Res. 247[1], 84-93. 25-2-1999.
8. **Andrejewski N, Punnonen EL, Guhde G, Tanaka Y, Lullmann-Rauch R, Hartmann D, von FK, Saftig P.** Normal lysosomal morphology and function in LAMP-1-deficient mice. J.Biol.Chem. 274[18], 12692-12701. 30-4-1999.
9. **Eskelinen EL, Schmidt CK, Neu S, Willenborg M, Fuertes G, Salvador N, Tanaka Y, Lullmann-Rauch R, Hartmann D, Heeren J, von Figura K, Knecht E, Saftig P.** Disturbed cholesterol traffic but normal proteolytic function in LAMP-1/LAMP-2 double-deficient fibroblasts. Mol.Biol.Cell 15[7], 3132-3145. 2004.
10. **Tanaka Y, Guhde G, Suter A, Eskelinen EL, Hartmann D, Lullmann-Rauch R, Janssen PM, Blanz J, von Figura K, Saftig P.** Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. Nature 406[6798], 902-906. 24-8-2000.
11. **Saftig P, Tanaka Y, Lullmann-Rauch R, von Figura K.** Disease model: LAMP-2 enlightens Danon disease. Trends Mol.Med. 7[1], 37-39. 2001.
12. **Eskelinen EL, Tanaka Y, Saftig P.** At the acidic edge: emerging functions for lysosomal membrane proteins. Trends Cell Biol. 13[3], 137-145. 2003.
13. **Eskelinen EL, Illert AL, Tanaka Y, Schwarzmann G, Blanz J, von Figura K, Saftig P.** Role of LAMP-2 in lysosome biogenesis and autophagy. Mol.Biol.Cell 13[9], 3355-3368. 2002.

14. **Beertsen W, Willenborg M, Everts V, Zirogianni A, Podschun R, Schroder B, Eskelinen EL, Saftig P.** Impaired phagosomal maturation in neutrophils leads to periodontitis in lysosomal-associated membrane protein-2 knockout mice. J.Immunol. 180[1], 475-482. 1-1-2008.
15. **Palokangas H, Mulari M, Vaananen HK.** Endocytic pathway from the basal plasma membrane to the ruffled border membrane in bone-resorbing osteoclasts. J.Cell Sci. 110 (Pt 15), 1767-1780. 1997.
16. **Akamine A, Tsukuba T, Kimura R, Maeda K, Tanaka Y, Kato K, Yamamoto K.** Increased synthesis and specific localization of a major lysosomal membrane sialoglycoprotein (LGP107) at the ruffled border membrane of active osteoclasts. Histochemistry 100[2], 101-108. 1993.
17. **Maeda H, Akasaki K, Yoshimine Y, Akamine A, Yamamoto K.** Limited and selective localization of the lysosomal membrane glycoproteins LGP85 and LGP96 in rat osteoclasts. Histochem.Cell Biol. 111[4], 245-251. 1999.
18. **Everts V, Korper W, Hoeben KA, Jansen ID, Bromme D, Cleutjens KB, Heeneman S, Peters C, Reinheckel T, Saftig P, Beertsen W.** Osteoclastic bone degradation and the role of different cysteine proteinases and matrix metalloproteinases: differences between calvaria and long bone. J.Bone Miner.Res. 21[9], 1399-1408. 2006.
19. **Everts V, Korper W, Jansen DC, Steinfort J, Lammerse I, Heera S, Docherty AJ, Beertsen W.** Functional heterogeneity of osteoclasts: matrix metalloproteinases participate in osteoclastic resorption of calvarial bone but not in resorption of long bone. FASEB J. 13[10], 1219-1230. 1999.
20. **Everts V, de Vries TJ, Helfrich MH.** Osteoclast heterogeneity: lessons from osteopetrosis and inflammatory conditions. Biochim.Biophys.Acta 1792[8], 757-765. 2009.
21. **Jansen ID, Mardones P, Lecanda F, de Vries TJ, Recalde S, Hoeben KA, Schoenmaker T, Ravesloot JH, van Borren MM, Van Eijden TM, Bronckers AL, Kellokumpu S, Medina JF, Everts V, Oude Elferink RP.** Ae2(a,b)-deficient mice exhibit osteopetrosis of long bones but not of calvaria. FASEB J. 23[10], 3470-3481. 2009.
22. **Giesen EB, Ding M, Dalstra M, Van Eijden TM.** Architectural Measures of the Cancellous Bone of the Mandibular Condyle Identified by Principal Components Analysis. Calcif.Tissue Int. 73[3], 225-231. 24-7-2003.
23. **Everts V, Korper W, Niehof A, Jansen I, Beertsen W.** Type VI collagen is phagocytosed by fibroblasts and digested in the lysosomal apparatus: involvement of collagenase, serine proteinases and lysosomal enzymes. Matrix Biol. 14[8], 665-676. 1995.

24. **de Vries TJ, Schoenmaker T, Beertsen W, van der NR, Everts V.** Effect of CD44 deficiency on in vitro and in vivo osteoclast formation. J.Cell Biochem. 94[5], 954-966. 1-4-2005.
25. **Bakker A, Klein-Nulend J.** Osteoblast isolation from murine calvariae and long bones. Methods Mol.Med. 80, 19-28. 2003.
26. **van Beek EM, de Vries TJ, Mulder L, Schoenmaker T, Hoeben KA, Matozaki T, Langenbach GE, Kraal G, Everts V, van den Berg TK.** Inhibitory regulation of osteoclast bone resorption by signal regulatory protein alpha. FASEB J. 23[12], 4081-4090. 2009.
27. **Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ.** Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93[2], 165-176. 17-4-1998.
28. **Wada T, Nakashima T, Hiroshi N, Penninger JM.** RANKL-RANK signaling in osteoclastogenesis and bone disease. Trends Mol.Med. 12[1], 17-25. 2006.
29. **Aoki S, Honma M, Kariya Y, Nakamichi Y, Ninomiya T, Takahashi N, Udagawa N, Suzuki H.** Function of OPG as a traffic regulator for RANKL is crucial for controlled osteoclastogenesis. J.Bone Miner.Res. 25[9], 1907-1921. 2010.
30. **Kariya Y, Honma M, Aoki S, Chiba A, Suzuki H.** Vps33a mediates RANKL storage in secretory lysosomes in osteoblastic cells. J.Bone Miner.Res. 24(10), 1741-1752. 2009.
31. **Ostrowski MC.** A new role for OPG: putting RANKL in its place. J.Bone Miner.Res. 25[9], 1905-1906. 2010.
32. **Zhou D, Li P, Lin Y, Lott JM, Hislop AD, Canaday DH, Brutkiewicz RR, Blum JS.** Lamp-2a facilitates MHC class II presentation of cytoplasmic antigens. Immunity. 22[5], 571-581. 2005.
33. **Yagi M, Ninomiya K, Fujita N, Suzuki T, Iwasaki R, Morita K, Hosogane N, Matsuo K, Toyama Y, Suda T, Miyamoto T.** Induction of DC-STAMP by alternative activation and downstream signaling mechanisms. J.Bone Miner.Res. 22(7), 992-1001. 2007.
34. **Dar A, Kollet O, Lapidot T.** Mutual, reciprocal SDF-1/CXCR4 interactions between hematopoietic and bone marrow stromal cells regulate human stem cell migration and development in NOD/SCID chimeric mice. Exp.Hematol. 34[8], 967-975. 2006.
35. **Honma M, Ikebuchi Y, Kariya Y, Hayashi M, Hayashi N, Aoki S, Suzuki H.** RANKL subcellular trafficking and regulatory mechanisms in osteocytes. J.Bone Miner.Res. 28[9], 1936-1949. 2013.

36. **Honma M, Ikebuchi Y, Kariya Y, Suzuki H.** Regulatory Mechanisms of RANKL Presentation to Osteoclast Precursors. Curr.Osteoporos.Rep. 12[1], 115-120. 2014.
37. **Settembre C, Ballabio A.** New targets for old diseases: lessons from mucolipidosis type II. EMBO Mol.Med. 5[12], 1801-1803. 2013.
38. **Kollmann K, Pestka JM, Kuhn SC, Schone E, Schweizer M, Karkmann K, Otomo T, Catala-Lehnen P, Failla AV, Marshall RP, Krause M, Santer R, Amling M, Braulke T, Schinke T.** Decreased bone formation and increased osteoclastogenesis cause bone loss in mucolipidosis II. EMBO Mol.Med. 5[12], 1871-1886. 2013.
39. **Settembre C, Fraldi A, Medina DL, Ballabio A.** Signals from the lysosome: a control centre for cellular clearance and energy metabolism. Nat.Rev.Mol.Cell Biol. 14[5], 283-296. 2013.